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DECLINE OF PSEUDOMONAS CITRI IN THE SOIL

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This investigation was undertaken primarily to determine whether or not the citrus-canker organism, *Pseudomonas citri* Hasse, is capable of persisting in the soil to such an extent as to make the soil an important medium in holding over or disseminating the organism.

EXPERIMENTAL METHODS

The work has been conducted in an isolated greenhouse near Washington, D. C. During the tests the soils were kept in ordinary 4- or 6-inch earthenware flower pots in duplicate, triplicate, or quadruplicate sets for each test. For the original inoculation of the soil it was found most satisfactory to use washings from potato cylinder cultures 2 to 10 days old. One such culture tube diluted with 200 cc. of water would give heavy inoculation in a 4-inch pot. The bacterial suspension was well mixed with the upper 3 inches of the soil, and samples were taken from this portion from at least three different points.

Because of the preponderance of more rapidly growing soil organisms, ordinary plating methods are inadequate for determining the abundance of *P. citri* in soil samples, and recourse was had to inoculation of punctured mature grapefruit leaves with graded dilutions of washings from the soil to be tested. The procedure was as follows: A sample of about 20 gm. of the soil was removed with a sterile spoon to a sterile Petri dish, and enough sterile distilled water was added to give an excess of about 10 cc. beyond saturation. This was well stirred, and 1 cc. of the soil solution was transferred to another Petri dish in which 9 cc. of sterile distilled water had been previously placed. The first washing described above is referred to as the 1/1 dilution in this paper, and the second as the 1/10 dilution. In a similar way dilutions of 1/100, 1/1,000 or beyond were made from the original soil wash water. Small wefts of sterile absorbent cotton were placed in each dish, one for each leaf to be inoculated. The grapefruit seedlings used were grown in 2, 2½, or 3 inch pots. They averaged 6 or 8 inches in height and had as a rule 8 to 12 leaves. Usually 5 leaves per plant were used for inoculation, and each leaf was punctured at 100 points. A simple device for making these punctures rapidly and accurately was improvised by inserting 10 sewing needles through a small cork stopper. This "punch" was readily sterilized by flaming the needles, was convenient to handle, made the punctures in a uniform group pattern, and thus contributed materially to the rapidity

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and accuracy of the work. A leaf was inoculated by wiping both under and upper surface of the freshly punctured portion with a cotton swab from a dilution dish, the swab being finally left on the upper surface. The inoculated plant was wrapped in paraffin paper, which served to retain moisture and to prevent accidental contamination from outside sources. Other plants were inoculated with pure cultures of *P. citri* as controls, and others were set up with the swabs merely wet with sterile water. The series were held at least a week in glass inoculation cases where conditions were near the optimum for canker development; later they were removed to the greenhouse benches. The first observations and records were made as a rule two to four weeks after inoculation. Final records were deferred until four to eight weeks after inoculation in order to insure the detection of any unusually slow development of infection such as occurred when the inoculum contained only a few organisms. The records show that between 90 and 95 per cent of the infections were apparent at the first observation and that no material increase was secured by holding beyond the second observation.

Variations of this method were tried out during its evolutionary development and to some extent in routine work as special considerations seemed to warrant. In many of the earlier series absorbent cotton wicks from small bottles of sterile water were placed in contact with the inoculation swabs on the leaves. This precaution to secure a prolonged moist condition proved to be unnecessary. An inoculum of mud paste, made by adding only a little water to the soil sample and applied with a backing of cloth or cotton as a sort of poultice over the punctured area, gave distinctly fewer infections than the soil solution in much greater dilutions. In cases where a large quantity of liquid inoculum could be prepared, a very effective method of inoculation was to dip the whole top of the test plant with its punctured leaves, keeping it submerged for an hour or longer, with several shakings during the period. In a few instances the test plants were so placed that the punctured leaves remained buried in the soil of the pots for a day or two. Tests were made of placing the plants under an air exhaust after soil water had been applied to their leaf surfaces. The soil solution was centrifuged to concentrate the canker organisms when they were very few, but this was without definitely satisfactory results. Still another method [1] employed was to atomize the leaves with sterile water, sift over them the rather dry soil to be tested, and keep the leaf surfaces moist for several days by holding the plants in a moist chamber and by repeatedly atomizing them with sterile water.

It was not apparent that any of the modifications of testing procedure could be relied upon to give a larger percentage of infections than the standard method, or to show the presence of *P. citri* when the standard method failed to give positive results.

[1] This method was first used by Miss Clara H. Hasse, of this office.

SENSITIVENESS OF DILUTION METHOD OF TESTING

In various tests involving several thousand plants, the standard testing method, which employs graded dilutions of the soil washing for inoculation on punctured grapefruit leaves, has proved reasonably sensitive in detecting the presence of viable *P. citri* in the soil. It is satisfactory for securing a rather definite idea of the relative numbers of this organism at the various times of sampling.

To test the efficiency of the method, dilutions in decimal series were made from a loopful of potato cylinder culture of *P. citri* distributed in the requisite number of cubic centimeters of sterile distilled water and were carried well beyond the vanishing point. One-cc. portions from each dilution were plated in beef agar for *P. citri* counts. Cotton swabs were dipped in the remainder of each dilution and applied to grapefruit leaves having 100 punctures each. Measurement showed these swabs to carry an average of 0.7 cc. of the liquid. The results of two independent tests are given in Table I.

TABLE I.—*Comparison between number of infections on grapefruit leaves and counts on poured plates, using graded dilutions of P. citri*

TEST A

	1/10,000	1/100,000	1/1,000,000	1/10,000,000	1/100,000,000	1/1,000,000,000	1/10,000,000,000
Average number of infections, 20 leaves tested	76	14	2	0.2	0	0	0
Average count for 1 cc. inoculum, 5 plates	22,300	2,500	300	32	1	0	0
Average number of organisms applied per leaf	15,600	1,750	210	22	.....	.....	.....
Average number of organisms per infection	205	125	105	110	.....	.....	.....

TEST B

Average number of infections, 16 leaves tested	69	9	1.4	0.13	0	0	0
Average count for 1 cc. inoculum, 6 plates	25,000	2,500	277	28	2.3	0.5	0.2
Average number of organisms applied per leaf	17,500	1,750	194	20	.....	.....	.....
Average number of organisms per infection	253	194	139	154	.....	.....	.....

The method apparently gives evidence of something like 30 organisms per cubic centimeter of inoculum, provided as many as 20 test leaves with 100 punctures each are used. The upper limit of sensitiveness would evidently be reached when numbers of bacteria are sufficient to infect practically all punctures, and diminution of sensitiveness would appear earlier. The ratio between infections per leaf and bacteria

applied is seen to be fairly uniform for the more critical lower ranges in both tests and lies between 1 to 100 and 1 to 200.

On the mature grapefruit leaves used for the tests there was practically no infection except at the freshly made punctures, and the counts are therefore free from errors that might have arisen from secondary spread if the unwounded tissue had been highly susceptible.

Two things are involved in the causation of infection by very dilute inoculum: (1) the chance for the organisms to reach the punctures and (2) the average number of organisms required to initiate infection successfully at a given point. In using cotton swabs, a considerable proportion of the organisms would be held at a distance from the leaf surface, and of those actually in the surface moisture film many would be at relatively great distances from punctures. On the other hand, motility of the organism and the possibility of rapid numerical increase by division would increase the chance for infection. As to the minimum number of *P. citri* organisms necessary to set up infection on reaching a given puncture, further careful experiments must be conducted before an opinion can be ventured.

PERSISTENCE IN VARIOUS TYPES OF SOIL

To secure as great diversity as possible with types of soil conveniently at hand the following kinds were selected: (1) stiff clay subsoil thrown out from an excavation several months previously; (2) leaf mold screened from ground surface in forest; (3) rotting compost of sod and manure thoroughly decayed; (4) garden soil, a clay loam of moderate fertility. Four-inch pots were used in duplicate for each soil type. The inoculum for each pot amounted to 10 cc. of a 2-day beef bouillon culture of *P. citri* mixed with about one-fifth of the washing from a 6-day potato cylinder culture, the whole being diluted to 100 cc. and evenly mixed with the upper 3 inches of soil in the pot. Inoculations were made on August 14, 1918. The pots were held in the greenhouse shaded from direct sunlight, and were given ordinary watering. Each percentage in Table II is based on the number of infections developed in 17 days in a total of 600 leaf punctures and represents the average of duplicate pots of each soil type.

TABLE II.—*Percentages of infection on grapefruit leaves inoculated with graded dilutions of solutions from four types of soil made at various intervals after the soil had been inoculated with P. citri*

Number of days between inoculation and sampling.	Clay subsoil.				Leaf mold.				Compost.				Garden soil.			
	1/1	1/10	1/100	1/1,000	1/1	1/10	1/100	1/1,000	1/1	1/10	1/100	1/1,000	1/1	1/10	1/100	1/1,000
0	28	52	15	0.3	55	63	13	2	35	16	8.3	3	60	33	32	2.3
2	9.2	11	0.8	0	17	8.7	0.8	0	11	22	57	4.7	60	33	28	14
5	.3	0	0	0	0.5	.2	0	0	38	32	5.2	.7	57	38	6.7	.5
9	0	0	0	0	.2	0	0	0	8.0	0.2	.2	0	3.5	0.7	0	.2
14	0	0	....	....	0	0	....	....	0	0	....	....	0	0	....	....

P. citri evidently decreased very rapidly in all these soils and apparently reached the vanishing point in all in less than 14 days. The rate of decrease was most rapid for the clay subsoil, slightly less so for the leaf mold, and distinctly slower for the compost and garden soil.

A second test was begun September 7, 1918, with new lots of soil from the same sources with the addition of well-washed sand from a creek bed, and a mixture of equal parts of the leaf mold and garden soil used in the earlier experiment. The initial inoculation was about 50 per cent heavier than in the preceding series. In order of rapidity of decrease clay subsoil proved again to be first, followed by leaf mold, sand, compost, garden soil, and mixture of leaf mold and compost. At the termination of this test, 14 days after inoculation, the red clay was the only one giving negative results; and the percentages for the leaf mold, compost, and garden soil were approximately those given for the ninth day in Table I. This longer persistence in the second test may reasonably be attributed to the higher initial inoculation of the soil.

In other experiments the following citrus soils from Florida were used: (1) from Orlando, intermediate between high and low pine soil types, unusually rich in humus; (2) similar to (1) but naturally poor; (3) similar to (2) but from a poorly drained spot; (4) from Bradentown, low pine land of low fertility; (5) from Bradentown, typical muck, extremely rich in humus; (6) from Winter Park, high hammock type; (7) from Winter Park, low hammock type. The samples, as a rule, reached the laboratory and were set up before becoming dry. The usual dilutions to 1/1,000 were run, but for brevity the percentages from the 1/1 dilution only are given in Table III. At the higher dilutions the commencement of decline was evident at the second sampling for all types, whereas this decline is not evident from the 1/1 figures of the table until the fifth or sixth day. The tests were made during September and October, 1918, in three distinct series, as indicated in the table. The second and third were conducted by Miss Clara H. Hasse, of this office, through whose courtesy the results have been furnished for this publication. The percentages are based on infection development from 600 punctures.

TABLE III.—*Percentages of infection on grapefruit leaves inoculated with 1/1 soil solution at various intervals after the soil had been inoculated with P. citri*

Series 1.				Series 2.			Series 3.		
Number of days between inoculation and sampling.	Soil 1.	Soil 2.	Soil 3.	Number of days between inoculation and sampling.	Soil 4.	Soil 5.	Number of days between inoculation and sampling.	Soil 6.	Soil 7.
0.....	88	93	80	0.....	31	62	0.....	92	83
2.....	90	93	88	3.....	61	98	.....	.....	.....
5.....	45	50	33	6.....	48	60	6.....	34	41
9.....	6.3	5.3	0.7	10.....	2.2	2.8	10.....	6.8	4.5
14.....	2.5	0	.5	16.....	.3	3.7	15.....	4.8	1.5
.....	.....	.....	.....	48.....	1.0	7.1	22.....	.2	.5
.....	.....	.....	.....	56.....	0	4.2	52.....	.2	0

There is a marked decline preceding the tenth day in all of the soils of Table III. But scattering infections are apparent over a much longer period, and no one of these soils could be safely declared free of *P. citri* at the times of discontinuance of the respective tests. It is a fact that regular watering of the pots was overlooked during the latter part of the longer tests, and the dry condition probably contributed to the long persistence. Special evidence on this point is given later in this paper.

Florida soils were also used in a number of other special tests, accounts of which follow throughout this paper.

Samples of soil from citrus plantings at Biloxi and Big Point, Miss., were artificially inoculated and tested at 6-day intervals for persistence of *P. citri*. The results were negative on the twelfth day and afterwards.

INFLUENCE OF DEGREE OF INITIAL SOIL INOCULATION

A series was set up September 16, 1918, using three degrees of inoculum, one five times and another one-fifth the usual medium degree. Unfortunately this series was discontinued on the twelfth day, just when the decline from the heavy inoculation was beginning to be pronounced. A second test was begun October 20, 1919. Greenhouse potting soil was used. The medium inoculation consisted of 0.4 of the washings from a potato cylinder culture for each of the duplicate pots. The heavy inoculation was 10 times this, and the light inoculation one-tenth. The pots were kept in the greenhouse, were shaded, and were given ordinary watering. Each percentage given in Table IV is based on 2,000 inoculated punctures.

TABLE IV.—*Percentages of infection on grapefruit leaves inoculated with graded dilutions of soil solution at various intervals after the soil had been inoculated with P. citri in different degrees*

Number of days between inoculation and sampling.	Heavy inoculation.				Medium inoculation.				Light inoculation.			
	1/1	1/10	1/100	1/1,000	1/1	1/10	1/100	1/1,000	1/1	1/10	1/100	1/1,000
0	62	23	5.8	0	9.8	4.8	0.3	0.6	6.0	0	0	0
2	33	30	5.5	1.0	9.1	3.0	.4	.2	1.0	0.1	0	0.05
4	11	3.5	1.6	.4	1.4	0	.05	0	0	.05	0	0
7	4.6	1.0	.4	0	0	0	0	0	0	0	0	0
9	.3	0	0	0	0	0	0	0	0	0	0	0
11	.05	0	0	0	0	0	0	0	0	0	0	0
14	.3	.05	0	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	.....	0	0	0	.....
23	.2	0	0	.....	0	0	.....	.....	0	0	.....	.....
30	0	.....	.....	.....	0	.....	.....	.....	0	.....	.....	.....

It is not understood why all the initial soil inoculations in this series turned out to be so far below the expected degree. What was intended for heavy soil inoculation ran considerably below that ordinarily used in other experi-

mental tests. At the same time it is probably as high as would be encountered in citrus plantings under infected trees; and the whole series may be regarded as representing high, medium, and low degrees of soil infection under natural conditions.

The differences are apparently not so much in rate of decline as in time required to reach the zero level from the different initial levels of inoculation.

INFLUENCE OF SOIL TEMPERATURE ON PERSISTENCE

The test for low temperature effect, series 1, which is reported in Table V, was made by exposing the inoculated potting soil to outdoor temperatures, beginning October 11, 1918. During the test the minimum daily readings ranged from 60° to 23° F., and the maximum daily readings from 83 to 58°. For moderate temperatures, exposure was made in the greenhouse, with daily means averaging about 15° higher than outside. Series 2 was begun October 20, 1919, using an incubator at 95° for the high range and the greenhouse for the moderate. The actual soil temperatures 2 inches below the surface were taken, the high temperature test ranging from 86° to 90° and the moderate from 68° to 72°. Percentages for series 1 are based on 600 inoculated punctures, and for series 2 on 2,000.

TABLE V.—*Percentages of infection on grapefruit leaves inoculated with graded dilutions of soil solutions at various intervals after the soil had been inoculated with P. citri and had been held at different temperatures*

Series 1.									Series 2.								
Days between inoculation and sampling.	Moderate temperature.				Low temperature.				Days between inoculation and sampling.	Moderate temperature.				High temperature.			
	1/1	1/10	1/100	1/1,000	1/1	1/10	1/100	1/1,000		1/1	1/10	1/100	1/1,000	1/1	1/10	1/100	1/1,000
0	....	....	66	7.2	...	...	53	12	0	9.8	4.8	0.3	0.6	5.8	1.4	1.3	0.3
3	....	97	62	9.5	...	68	60	42	2	9.1	3.0	.4	.2	0.1	0	0	0
7	....	3.3	3.3	1.8	...	...	73	50	4	1.4	0	.05	0	.3	0	0	0
10	24	2.0	.3	1.7	45	90	37	14	7	0	0	0	0	0	0	0	0
15	0.3	.3	0	....	24	90	25	....	9	0	0	0	0	0	0	0	0
19	....	....	[a]1.3	....	...	...	[a]21	....	11	0	0	0	0	0	0	0	0
28	....	....	[a].2	....	...	...	[a]3.1	....	14	0	0	0	0	0	0	0	0
36	....	....	[a]0	....	...	...	[a]0	....	18	0	0	0	0	0	0	0	0
42	....	....	[a]0	....	...	...	[a]0	....	23	0	0	0	0	0	0	0	0

[a] Inoculated by dipping plant top in liquid.

There is a very evident retardation of the rate of decline at the lower temperatures. A second series of October 23, 1918, confirms this for a still lower range of temperature. The higher temperatures seem to accelerate the decline, but the unfortunate low initial inoculation of the soil requires a repetition of the test. At the time of handling series 1, the influence of soil dryness in prolonging persistence had not been determined,

and too little attention was given to watering the pots regularly during the latter part of the experiment. But the outside pots retained moisture much better than those inside, and any difference would have been against longer persistence in them.

INFLUENCE OF SOIL MOISTURE ON PERSISTENCE

A test was begun September 2, 1918, using ordinary potting soil. Inoculation was with a mixture of beef bouillon and potato cylinder cultures. One set of duplicate pots was kept near the saturation point by watering thoroughly every other day. A second lot was restored at each watering to the halfway point between saturation and the original air-dry condition of the soil. The third set was left unwatered. The percentages in Table VI are based on 600 inoculated punctures.

TABLE VI.—*Percentages of infection on grapefruit leaves inoculated with graded dilutions of soil solution at various intervals after the soil had been inoculated with P. citri and had been held at three moisture contents*

Number of days between inoculation and sampling.	Soil continuously saturated.				Soil moderately watered.				Soil air dry.			
	1/1	1/10	1/100	1/1,000	1/1	1/10	1/100	1/1,000	1/1	1/10	1/100	1/1,000
0	62	70	77	68	68	55	80	47	43	80	85	57
1	54	23	38	32	57	38	18	43	55	83	47	42
3	53	72	37	8.7	67	60	45	21	67	78	71	70
5	48	55	23	11	75	48	43	4.3	65	53	7.2	7
7	2.2	0.8	0.2	0	10	4.2	1	0	11	3.3	1.2	0
12	1.5	.7	.3	0	0.3	.2	0	0	1.8	4.5	.8	0
17	0	.3	.....	.....	.2	0	.....	.....	3.5	.2	.....	.....

The foregoing test shows no very pronounced or definite differences in rate of decrease. The slight differences tend toward lag with decrease of moisture, the moderately watered soil showing possibly less rapid decline than the saturated, and the air-dry soil showing still greater retardation.

Further tests of moderately wet soil as compared with dry were made at different times with three lots of Florida soil and are reported in Table VII. The soil for series 1 was from a "sand-soak" spot at Estero, Fla.; for series 2, from high pine land near Leesburg, Fla.; and for series 3, from intermediate pine land at Orlando, Fla. These tests were made by Miss Clara H. Hasse, of this office, during October and November, 1918, and through her courtesy are presented here. Only the 1/1 dilutions are included in Table VII, since in each series the results from higher dilutions were in accord with these. The percentages are based on inoculation of 600 punctures.

The first series indicates distinctly a retarded decline and prolonged persistence in the dry soil. The second series, with another type, shows no decided difference between the wet and dry. In the third series the initial decline was more rapid in the dry than in the wet soil.

TABLE VII.—*Percentages of infection on grapefruit leaves inoculated with 1/1 solutions of three Florida soils at various intervals after the soils had been inoculated with P. citri and had been held at two moisture contents*

Number of days between inoculation and sampling.	Series 1.		Series 2.		Series 3.	
	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.
0	98	98	100	97	95	100
3	98	100	100	95	86	19
6	83	74	77	90	8.8	0.5
9	82	94	90	64	5.7	.7
14 or 15 [a]	9.7	93	63	23	0	.3
21	6.3	40	1.8	1.7	.....	.....
44 or 43	0	18	0	0	.....	.....
50 to 54	0	0	0	0	0	0

[a] Where two numbers appear for days they indicate slight differences in the sampling periods for the several series.

In Table VIII two series, one set up June 12 and one July 8, 1919, are compared. Both were with soil from Orlando, Fla., of the same type but collected at different times. The inoculum for each 6-inch pot in the two series was from four potato cylinder cultures. The first series was kept well watered. The second was dried overnight after the original inoculation and kept air-dry thereafter. The percentages for the first series are averaged for four similar pots and are based on 4,000 inoculated punctures; those for the second are for three pots and are based on 3,000 inoculated punctures. These series were set up and conducted for approximately the first two months by Miss Clara H. Hasse.

TABLE VIII.—*Percentages of infection on grapefruit leaves inoculated with graded dilutions of soil solution at various intervals after the soil had been inoculated with P. citri and had been held at two moisture contents*

Number of days between inoculation and sampling.	Series 1, moist soil.				Series 2, dry soil.			
	1/1	1/10	1/100	1/1,000	1/1	1/10	1/100	1/1,000
0	59	73	70	60	94	97	98	73
2	66	93	87	92	22	7.5	2.4	0.4
4	40	17	50	13	11	2.0	2.9	.1
7, 6 [a]	44	44	11	3.9	2.6	2.1	.4	0
9, 8	17	14	2.5	1.6	3.9	2.1	.5	0
11, 10	13	8.3	3.0	1.0	23	4.6	.8	0
13	2.1	2.4	.6	.02	30	4.0	.9	.3
18	2.8	1.2	.3	.2	16	2.7	.4	12
21, 23	.6	.4	.03	0	32	9.6	.9	.3
33, 34	0	.03	0	.....	20	1.7	.1	0
40, 43	.03	0	0	.....	3.7	1.1	.3	.....
52, 54	0	.03	0	.....	.6	.2	.03	.....
60, 62	0	0	0	.....	1.7	.5	.....	.....
69, 71	0	0	.....	.....	1.5	.6	.....	.....
78, 80	0	.....	.....	.....	3.0	.9	.....	.....
88, 90	0	.....	.....	.....	.4	.....	.....	.....
97, 99	0	.....	.....	.....	.1	.....	.....	.....
106, 108	0	.....	.....	.....	.1	.....	.....	.....
116, 118	0	.....	.....	.....	.05	.....	.....	.....
125, 133	0	.....	.....	.....	.1	.....	.....	.....
..., 166	.....	.....	.....	.....	.1	.....	.....	.....

[a] Where two numbers appear for days the first applies to series 1 and the second to series 2.

Heavy initial inoculation, frequent samplings over a long period, and inoculation at each sampling of 40 or 30 grapefruit leaves with 100 punctures each for the respective series render the results in these series especially noteworthy. In the moist series, after the fourth day, one notes a general equality of percentages on diagonals extending downward and to the left from any of the 1/1,000 figures. For example, the 1/1,000 dilution on the fourth day, the 1/100 on the seventh, the 1/10 on the ninth, and the 1/1 on the eleventh are approximately the same, indicating a nine-tenths loss in actual numbers in the soil for each sampling interval as compared with the preceding one; and this seems to hold true until the fortieth day. It may be explained that the moist series suffered much from the dropping of leaves heavily infected from the early samplings, and the resulting figures are somewhat erratic.

In the dry series there is a decided drop following the initial drying immediately after inoculation. Then follows a slow decline followed by an inexplicable increase between the tenth and thirty-fourth days. Afterwards there is an extremely gradual decline, if any, extending to the one hundred and sixty-sixth day.

On October 27, 1919, the one hundred and twelfth day of the test, a portion of the soil was removed from each of the three dry pots and moistened with sterile distilled water. The following tabulation shows the results of inoculation tests made from these moistened lots in comparison with the original dry soil. Two thousand punctures were inoculated from each lot of soil, making 6,000 for each test of moistened or of dry soil. The figures are total infections from 6,000 punctures.

	Date of sampling.						
	Oct. 29.	Oct. 31.	Nov. 3.	Nov. 5.	Nov. 7.	Nov. 10.	Nov. 17.
Dry soil	2	6	3	8	9	0	6
Moistened soil	0	0	0	0	0	0	0

The application of sterile distilled water seemingly resulted in prompt and complete extinction of *P. citri* in this dry soil which had constantly shown the presence of at least small numbers of the organism during almost four months. A repetition of the test, begun November 14, 1919, confirms these results.

That this extinction was not due to any toxic property peculiar to the distilled water was shown by a second test begun December 13, 1919, in which spring water and deep well water were used for wetting the soil. Tests on the third and seventh days were negative for all lots of moistened soil, while the dry soil continued to show the usual trace of *P. citri*.

That rate of drying would have an influence on the residuum of *P. citri* is to be expected and probably accounts for some of the irregularities

already noted in the behavior of the dry soil series when no control was exercised over the rate of drying. In a preliminary test, comparisons were made of the infective power of soil samples similarly inoculated and air-dried with different rates of rapidity at approximately the same rather warm temperature. A sample dried in less than one day gave 1.5 per cent infection, one dried in two days gave 0.1 per cent infection, and one dried in seven days gave no infection in tests made in each case immediately after drying.

An extended test of persistence in air-dry soil was made by Miss Clara H. Hasse. On October 22, 1918, soil from Winter Park, Fla., was heavily inoculated and dried as quickly as possible, in about one hour. Tests for infectiveness were made by several methods, usually by dusting the dry soil over punctured leaves which were atomized with water, the plants being later held in moist chambers. The total punctures inoculated in each test ranged from 600 to 5,000. The following percentage results were obtained:

	Date of sampling.									
	Oct. 22, 1918.	Oct. 25, 1918.	Oct. 28, 1918.	Nov. 1, 1918.	Nov. 8, 1918.	Jan. 2, 1919.	June 11, 1919.	Aug. 8, 1919.	Sept. 23, 1919.	Dec. 26, 1919.
Percentage of infection	94. 1	68. 3	8. 5	2. 3	1. 5	0. 1	0. 24	0. 22	0. 48	0. 05

On December 22, 1919, a portion of this soil was moistened with tap water from a deep well and was tested on the fourth and seventh days in comparison with the part remaining dry. In these tests the moistened soil gave no infection, while the dry soil continued to show traces.

PERSISTENCE IN SOILS MADE ARTIFICIALLY ALKALINE AND ACID

Greenhouse potting soil was used in 6-inch pots. Duplicate pots were watered each with 400 cc. of water containing 1.6 cc. sulphuric acid. Two pots were watered with 400 cc. of water containing 224 cc. clear lime water prepared by slaking 25 gm. quicklime and making up to 1,000 cc. A titration test showed this lime water to be sufficient to neutralize the quantity of acid applied to the other pots. A third pair of pots was watered with 400 cc. distilled water. After standing three days all pots were equally inoculated with *P. citri*. On each sampling date litmus paper tests of the 1/1 soil washings were made, and such small amounts of lime water or diluted acid were added as seemed necessary to maintain approximately the original distinct acidity in one set and distinct alkalinity in the other. The watering of all sets was equalized. The percentage results given in Table IX are based on infections out of 2,000 inoculated punctures.

TABLE IX.—*Percentages of infection on grapefruit leaves inoculated with graded dilutions of three soil solutions at various intervals after the soils had been treated with lime water, dilute sulphuric acid, and distilled water, respectively, and inoculated with P. citri*

Number of days between inoculation and sampling.	1. Alkaline soil.				2. Normal soil.				3. Acid soil.			
	1/1	1/10	1/100	1/1,000	1/1	1/10	1/100	1/1,000	1/1	1/10	1/100	1/1,000
0	80	95	70	33	93	100	46	26	16	80	50	24
2	69	84	67	49	83	70	46	23	7.5	22	3.8	1.5
4	56	46	38	8.9	41	40	30	3.0	.6	.5	.5	0
7	9.1	5.1	2.9	.6	3.8	2.1	.6	.2	0	0	0	0
9	2.7	.4	.2	.1	.5	.2	0	0	0	0	0	0
11	.1	0	0	.05	0	0	0	0	0	0	0	0
14	0	.3	0	0	.05	0	0	0	0	0	0	0
18	.2	.5	0	.05	0	0	0	.05	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	.....	.....	0	0	.....	.....	0	0	.....	.....
37	0	.....	.....	.....	0	.....	.....	.....	0	.....	.....	.....
46	0	.....	.....	.....	0	.....	.....	.....	0	.....	.....	.....

This preliminary and very artificial series indicates a slight retardation of decline in the alkaline soil and a distinct acceleration in the acid soil. In the latter, one notes the low infection percentages for the 1/1 dilution as compared with the 1/10 of the same series, or with the 1/1 of the other two series. While there is quite generally a tendency for the 1/1 dilution to give unexpectedly low results, the present instance suggests that the rather high acidity of the first wash water vehicle may play a part here in preventing infection. This matter calls for further experimentation. Since the tendency of most citrus soils is toward acidity, the evidence presented in Table IX is reassuring as to the decline of *P. citri* in such soils, notwithstanding the very unnatural conditions of the experiment.

PERSISTENCE DEEP IN THE SOIL

The tests for downward penetration were made by placing partially dry soil in open pasteboard cylinders 3 inches in diameter and watering the surface with a strong *P. citri* suspension until the whole was saturated. Sections were made at proper intervals and samples taken with proper precautions from the axis of the soil column. For vertical ascent the cylinders were placed in a shallow pan containing the suspension of *P. citri*.

In an 8-inch column of Florida sandy soil sampled at 2-inch intervals on November 20, 1918, downward penetration was shown to be very uniform throughout. A 15-inch column of greenhouse potting soil was tested October 1, 1919, with similar practically uniform penetration, as shown by sampling at 3-inch intervals.

In Florida soil tested for vertical ascent, the capillary rise was 6 inches during four hours. Two-inch samplings showed *P. citri* to be uniformly distributed.

While testing experimental methods, it was found that the organism is readily carried in the capillary current along an absorbent cotton wick at least 10 inches.

The indication that *P. citri* may readily penetrate deep into the soil raises the question of whether conditions deep in the soil may influence the persistence of the organism differently from those near the surface. A test was made by burying 4-inch pots of inoculated potting soil in large containers, so that the pots were completely surrounded by 8 inches of soil. Samplings were made at approximately 5-day intervals. No decided difference was noted between the buried pots and similarly inoculated ones held on the greenhouse bench.

PERSISTENCE IN AUTOCLAVED SOIL

Greenhouse potting soil in 4-inch pots was autoclaved July 15, 1918, for one hour with steam at 12 pounds pressure. When the soil was cold four autoclaved pots were inoculated, as well as four others containing similar soil not autoclaved. The percentage results in Table X are based on infection out of 1,200 inoculated punctures.

TABLE X.—*Percentages of infection on grapefruit leaves inoculated with graded dilutions of solutions from unautoclaved and autoclaved soils at various intervals after the soils had been inoculated with P. citri*

Number of days between inoculation and sampling.	Unautoclaved soil.				Autoclaved soil.			
	1/1	1/10	1/100	1/1,000	1/1	1/10	1/100	1/1,000
0	73	73	16	0.9	44	3.4	6.2	0.3
4	60	35	7.8	3.7	60	68	13	15
9	0.9	0.7	.1	0	28	17	75	40
14	0	0	0	0	9.5	4.3	.8	.7
18	.1	.2	0	0	9.2	13	9.4	1.3
24	0	.1	0	0	1.9	1.9	2.1	.2
29	0	0	0	0	.8	.3	.3	.2
35	0	0	0	0	.4	.1	0	.1
44	0	0	.....	.....	0	0	.....	.....

The pots were kept on the greenhouse bench, each covered with paper. No special precautions were adopted to insure continued sterility in the autoclaved pots, if indeed the original steaming was sufficient for complete sterilization. Platings on agar at the end of the test showed miscellaneous bacteria in these pots in apparently as great numbers as in the unautoclaved ones. The autoclaved soil shows a decided lag in the decline of *P. citri*. A second series run two months later confirms this result.

PERSISTENCE IN WATER

Water was held in cotton-stoppered flasks in 200-cc. quantities. Water from a local spring was used in comparison with distilled water. Unfortunately the flasks of autoclaved distilled water became contaminated,

as was shown by Petri dish platings soon after the series was begun, and the results from them are not included in the tabulation. The percentages in Table XI are based on infection in 1,500 punctures.

TABLE XI.—*Percentages of infection on grapefruit leaves inoculated with graded dilutions of distilled water, autoclaved spring water, and unautoclaved spring water at various intervals after the water had been inoculated with P. citri*

Number of days between inoculation and sampling.	Distilled water.				Spring water, autoclaved.				Spring water, not autoclaved.			
	1/1	1/10	1/100	1/1,000	1/1	1/10	1/100	1/1,000	1/1	1/10	1/100	1/1,000
0	100	97	43	14	97	87	51	8.2	100	96	58	13
2	0.1	0	.0	0	72	8.2	5.8	.3	1.7	0.5	0.5	0
4	0	0	0	0	85	28	5.1	.8	0	0	0	0
7	0	0	0	.....	90	69	12	.....	0	0	0	.....
11	0	0	.....	.....	58	25	.....	.....	0	0	.....	.....
16	0	.....	.....	.....	34	.....	.....	.....	0	.....	.....	.....
21	0	.....	.....	.....	44	.....	.....	.....	0	.....	.....	.....
30	0	.....	.....	.....	40	.....	.....	.....	0	.....	.....	.....

Two other series confirmed the very rapid decline noted above in either distilled or ordinary surface water when it is nonsterile. Contrasted with this is the long persistence of moderately reduced numbers of the organism in the sterilized spring water.

The question is sharply raised, does autoclaving promote the persistence of *P. citri* in soil or water by destroying something that is deleterious or by producing something that is favorable? Autoclaving, in general, has its greatest effect in destroying the organic fauna and flora of the medium, and a subsidiary one in modifying the nutritive materials contained in it. The supposition that starvation may be the cause of the normal decline and that autoclaving the soil supplies enough available nutriment for a greatly prolonged persistence does not seem reasonable because of the disproportion between the changes that could possibly be brought about by autoclaving and the effects observed on *P. citri* persistence. Furthermore, this supposition of starvation is not adequate to explain the extinction of *P. citri* in air-dry soil when moistened.

INHIBITORS

The deleterious effects of organisms on the development of *P. citri* is frequently observed in poured plates when fungus or bacterial contaminators entirely inhibit the development of *P. citri* for considerable distances from their limits of growth. The behavior of some of these inhibitors has been made the subject of special preliminary study.

A series of plates was prepared September 13, 1919, from beef agar rather heavily and uniformly inoculated with *P. citri*. When hard, they were inoculated in addition with a bacterium, designated inhibitor A, previously obtained from a chance contamination on a poured plate. On some plates two streaks of the inhibitor were made at right angles

across the plates; in others it was planted at the center and at four spots near the circumference; in still others it was planted abundantly over the plate. Seven days later *P. citri* was seen growing in triangular areas between the limbs of the crosses, in isolated patches with concave borders between the spots, and not at all on the plates with numerous colonies of the inhibitor. It appeared only where the distance was at least 15 to 18 mm. from the nearest border of an inhibiting colony. A hand lens and the low power of the microscope brought within range of vision two successive graded zones each about 3 mm. wide of smaller *P. citri* colonies edging the clearly visible areas. The average distance from the edge of the inhibiting colony to the *P. citri* colonies of microscopic size was about 10 mm. Repeated attempts to cultivate *P. citri* from bits of agar from this clear 10-mm. zone failed, although the abundance of the original inoculation would have made it easy to recover the organism at any point, if it were still alive. It was recovered in culture from the microscopic and the clearly visible zones, and no extension of the killing effect could be determined after a further lapse of seven days, during which time there was no apparent growth of the inhibiting colonies.

The testing of some 40 miscellaneous soil bacteria and fungi on beef agar plates by the streak or the spot method showed about one-fourth of the number to have some degree of inhibiting effect, while three seemed to stimulate or accelerate the development of *P. citri* colonies, at least at the beginning of their development.

On other media the effects of certain of these inhibitors differed from those exhibited on beef agar, the inhibiting effect being reduced or entirely lost on certain media.

Tests in the soil itself must be conducted before definite statements can be made as to the part such potential inhibitors or destroyers actually play in the decline of *P. citri* under soil conditions. However, the hypothesis that the deleterious effects on *P. citri* are brought about by certain organisms in the soil is in harmony with the experimental evidence thus far obtained and seems to be a reasonable explanation of the phenomenon.

It seems reasonable to suppose that *P. citri* can persist in dry soil partly at least because of suspended activity of deleterious organisms, and that the addition of water makes possible a renewal of their unfavorable activity.

INFECTION OF GRAPEFRUIT ROOTS BY P. CITRI

The question naturally arises as to whether roots of citrus species are highly susceptible to citrus-canker infection. The following tests bear on this point.

On May 20, 1918, eight pots of soil were inoculated heavily with *P. citri* culture and planted with grapefruit seed, about 50 per pot. Eight other pots were similarly prepared without inoculation. In another set both seed and soil were inoculated, and in still another only the seeds were

inoculated. After two months the seedlings in all had made good growth, and there was no evidence of citrus-canker lesions on any part of the plants. In the light of present knowledge it would not have been expected that a single soil inoculation at the time of planting would persist long enough to become very effective.

On July 10, 1918, a series of pots was planted with grapefruit seed and given frequent waterings with *P. citri* suspension, on July 10, 13, 17, 20, 24, 27, and August 2. By this time the seedlings had emerged above ground, and before each subsequent watering several cuts were made through the soil with a knife to produce root wounds. Further applications of *P. citri* suspension were made August 5, 8, 10, 14, and 16. On August 31 the seedlings were removed, washed, and examined with a hand magnifier. No canker lesions were apparent on any part of the 40 plants thus examined. A test performed on grapefruit leaves with soil from these pots which had received 12 applications of heavy inoculum at close intervals gave negative results.

Direct inoculation of the roots of potted grapefruit seedlings was made as follows: On July 27, 1918, potted plants were selected with vigorous roots of about $^3/_{16}$ inch diameter extending ½ to 3 inches through the drainage holes of the pots. These roots were punctured at 10 points each, wrapped in cotton wet with *P. citri* suspension, and later placed in flats of moist, clean sand. Two weeks later infection was 40 per cent. Microscopic sections showed typical canker lesions involving the cortex. Pure cultures of *P. citri* were readily obtained by plating, and grapefruit leaves were infected therefrom. Four months later no extension of infection was apparent on the roots, most of them having continued their growth to all appearance normally. In several, however, the roots were broken at old lesions apparently following secondary decay. The plants as a whole had not suffered.

The indications are that young grapefruit roots are not readily infected except through direct wound inoculation and that the plants do not suffer from a moderate number of lesions so produced.

SUMMARY

(1) The method of using graded dilutions of soil washings for inoculating punctured grapefruit leaves proved satisfactory for indicating the relative abundance of *P. citri* in the soil at times of sampling.

(2) Tests on many types of soil, including representative ones from citrus regions, show a very rapid decline of *P. citri* in all.

(3) This decline was retarded slightly by rendering the soil alkaline with lime water or by lowering its temperature, and more decidedly by withholding water or by previous sterilizing with steam.

(4) An extremely long persistence, in very small numbers, is noted in soil held in air-dry condition; but the organism seemingly suffers prompt extinction when water is again added.

(5) The decline is accelerated decidedly by the addition of dilute sulphuric acid or by a moderate rise in temperature.

(6) *P. citri* may easily penetrate the soil to depths ordinarily cultivated, but the normal decline seems to occur at such depths.

(7) In water the decline is more rapid than in soil. Previous sterilizing of the water has a decided effect in prolonging persistence.

(8) Certain bacteria found commonly in soils have a marked deleterious effect on *P. citri* in artificial culture media both by inhibiting growth and by killing.

(9) The presence of such deleterious organisms in soils would probably be concerned in producing a decline of *P. citri*.

(10) Young roots of grapefruit seedlings seem not to be readily infected by *P. citri* except through wounds.

CONCLUSION

The main question at issue is whether or not *P. citri* can persist in the soil to a sufficient degree or for a long enough time to be a source of danger in the dissemination or holding over of the citrus-canker disease. The experimental evidence shows clearly that the organism undergoes a rapid and continuous decline in numbers under soil conditions that would obtain in agricultural practice. As a rule, this decline reaches the vanishing point for *P. citri* in about two weeks by the test methods employed, and it is only reasonable to suppose that the downward trend continues rapidly in such cases to absolute extinction. The potential ability of certain soil organisms to destroy *P. citri*, as shown in certain artificial culture media, lends weight to this latter supposition. Even where long-time persistence has been induced experimentally, the conditions necessary to bring it about are too extreme to make a duplication probable under natural conditions. Furthermore, the experimental methods employed for testing the infectiveness of the soil are many times more severe than would obtain under most favorable natural conditions for the spread of infection from soil to plants. All these considerations suggest that agricultural soils probably can not long retain a dangerous possibility of disseminating the citrus-canker organism.